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DESCRIPTION

PEPTIDE DERIVED FROM HEPATITIS C VIRUS

Technical Field

The present invention relates to a hepatitis C virus
(HCV) peptide, which is useful for diagnosing hepatitis
C virus infection and treating hepatitis C.

More particularly, the invention relates to an HCV
peptide, a polypeptide comprising an HCV peptide, a
nucleotide encoding an HCV peptide or the polypeptide,
an antibody or a substance with an antibody-like activity
that recognizes the peptide or polypeptide, a vector
comprising the nucleotide, a method of inducing a
cytotoxic T cell with the HCV peptide or the polypeptide,
a method of detecting hepatitis C virus and a method of
diagnosing, preventing or treating hepatitis C, a method
of predicting the prognosis of hepatitis C using the HCV
peptide, the polypeptide, the nucleotide, or the
antibody or the substance with an antibody-like activity,
as well as a pharmaceutical composition for treating
hepatitis C comprising the HCV peptide, the polypeptide,
the nucleotide, or the antibody or the substance with
an antibody-like activity.

Background Art

Hepatitis caused by viruses includes hepatitis A mainly caused by oral infection with virus and hepatitis B caused by infection through blood, and hepatitis C mainly caused by infection through blood transfusion. The hepatitis C is one of the diseases that may develop into a chronic disease resulting in hepatic cirrhosis or liver cancer at an extremely high risk.

Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to the flavivirus family, and 2 million or more in Japan, and 170 million around the world are said to be infected with hepatitis C virus.

A combination therapy with interferon and ribavirin has been developed for treating hepatitis C, however, it is effective only in 30 to 40% of patients, and effective therapy is not available for the majority of the patients. Accordingly, there has been a strong social demand to immediately develop diagnostic and therapeutic tools which are safe, effective and simple to use at low costs.

There is evidence showing that both cellular and humoral immune responses play a primary role in inhibiting HCV infection (WO 89/04669). A number of studies have been conducted over the past 10 years to find an HCV peptide with high immunogenicity, and many

HCV peptides have been discovered that can induce a
cellular or humoral immune response (Hunziker et al.,
Mol Immunol. 2001 Dec; 38(6): 475-84). However, any of
these peptides is not clinically effective. There is no
effective immunotherapeutic means for preventing and
treating hepatitis C even at present, which is believed
to be primary due to the poor immunogenicity of these
peptides.

In addition, the present inventors have already
reported that an antigenic peptide recognized by some
cytotoxic T lymphocytes (CTLs) has an ability to induce
both cellular and humoral immune responses both in vitro
and in vivo (Gohara et al., J Immunother. 2002 Sep-Oct;
25(5): 439-44, Mine et al., Clin Cancer Res. 2001 Dec;
7(12): 3950-62, Tanaka et al., J Immunother. 2003
Jul-Aug; 26(4): 357-66, Mine et al., Cancer Sci. 2003
Jun; 94(6): 548-56).

An HCV peptide recognized by both cellular and
humoral immune responses is expected to have higher
immunogenicity than a peptide recognized only by a
cellular immune response.

A new approach to meet such a social demand is to
identify an HCV peptide which can induce both cellular
and humoral immunity, since accumulating findings
demonstrated that such a peptide would have much higher

immunogenicity than a peptide inducing only a humoral immunity.

Thus, it will be advantageous to identify such a peptide and to examine whether such a peptide is a candidate for a novel therapeutic and diagnostic tool for HCV infection.

Accordingly, the present inventors have examined whether IgG reactive to a peptide recognized by HLA-A2- and HLA-A24-restricted cytotoxic T lymphocytes (CTLs) can be detected in the serum of a patient infected with HCV, and found that IgG specific for certain CTL epitopes is detected in the majority of the patients infected with HCV regardless of their HLA class I type or the HCV genotype. From this result, they found that such a peptide may lead to novel preventive and therapeutic tools utilizing the peptide.

Accordingly, an object of the invention is to provide an HCV peptide which is recognized by cellular and humoral immunity and is highly immunogenic.

Disclosure of the invention

The present invention provides a peptide derived from hepatitis C virus, comprising an HLA-binding motif in its sequence and is recognized by an antibody present in the blood of a patient infected with hepatitis C virus.

In a preferred embodiment, the peptide derived from hepatitis C virus has an amino acid sequence represented by any one of SEQ ID NOS: 1 to 8, 16, 20 and 38 of the sequence listing, or has an amino acid sequence having a homology of at least 70% with the amino acid sequence of the HCV-derived peptide, and has a property of being recognized by an HLA-A2- or HLA-A24-restricted cytotoxic T cell.

Another aspect of the invention provides a polypeptide comprising the peptide derived from hepatitis C virus. In a preferred embodiment, the invention also provides a polypeptide having an amino acid sequence having a homology of at least 70% with the amino acid sequence of the polypeptide, and a polypeptide further having a property of being recognized by an HLA-A2- or HLA-A24-restricted cytotoxic T cell.

Yet another aspect of the invention provides a nucleotide encoding the peptide derived from hepatitis C virus or the polypeptide.

Still another aspect of the invention provides an antibody or a substance with an antibody-like activity capable of immunologically recognizing the peptide derived from hepatitis C virus or the polypeptide.

In still another aspect, the invention provides a vector comprising the above-mentioned nucleotide.

Still another aspect of the invention provides a method of inducing a cytotoxic T cell by using the nucleotide encoding the peptide derived from hepatitis C virus or the polypeptide.

In still another aspect, the invention provides a method of detecting hepatitis C virus using the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity.

Another aspect of the invention provides a method of diagnosing a disease associated with HCV infection such as hepatitis C using the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity.

In another aspect, the invention provides a method of treating a disease associated with HCV infection such as hepatitis C using the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity.

In still another aspect, the invention provides a pharmaceutical composition comprising as an active ingredient the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity. In a preferred embodiment, the pharmaceutical composition is a hepatitis C virus vaccine.

In another aspect, the invention provides a method of predicting the prognosis of a disease associated with HCV infection such as hepatitis C using the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity.

In still another aspect, the invention provides a kit for diagnosing hepatitis C or predicting the prognosis of hepatitis C comprising the peptide derived from hepatitis C virus, polypeptide, nucleotide or antibody or substance with an antibody-like activity.

Brief Description of the Drawings

Fig. 1 is a graph showing the results of measuring the IgG level in the blood of a patient infected with HCV by the ELISA method according to the present invention.

Fig. 2 is a graph showing the results of measuring the IgG level in the blood of patients infected with HCV by the ELISA method according to the present invention.

Fig. 3 is a graph showing the experimental results of absorption and elution of a representative example of an antibody reactive to C-35 peptide present in the serum of the patient shown in Fig. 1 according to the present invention.

Fig. 4 is a graph showing the results of CTL

induction with 6 types of HLA-A24 binding peptides (Nos. 3, 12, 13, 25, 32 and 38) derived from HCV according to the present invention.

Fig. 5 is a graph showing the results of CTL induction with HLA-A2 binding peptides (Nos. 40 to 57) derived from HCV according to the present invention.

Fig. 6 is a graph showing representative results of the cytotoxic activity of peptide-stimulated PBMC in a ^{51}Cr release assay according to the present invention.

Fig. 7 is a graph showing representative results of the cytotoxic activity of peptide-stimulated PBMC in a ^{51}Cr release assay according to the present invention.

Fig. 8 is a graph showing the HLA class I restriction of the cytotoxicity of peptide-stimulated PBMC in an inhibition assay with an anti-CD8 monoclonal antibody according to the present invention.

Fig. 9 is a graph showing detection of anti- peptide IgG in the sera of patients infected with HCV.

Fig. 10 is a graph showing detection of anti-peptide IgG in the sera of patients infected with HCV.

Fig. 11 is a graph showing the results of adsorption test analyzing the specificity of anti-peptide IgG present in the sera of patients infected with HCV.

Fig. 12 is a graph showing the results of elution test analyzing the specificity of anti-peptide IgG in

the sera of patients infected with HCV.

Fig. 13 is a graph showing IFN-γ production by peptide-stimulated PBMC.

Fig. 14 is a graph showing IFN-γ production by peptide-stimulated PBMC.

Fig. 15 is a graph showing the cytotoxicity of peptide-stimulated PBMC.

Fig. 16 is a graph showing the cytotoxicity of peptide-stimulated PBMC.

Fig. 17 is a graph showing the CTL activity of peptide-stimulated PBMC against NS5A-expressing cells.

Fig. 18 is a graph showing the CTL activity of peptide-stimulated PBMC against NS5A-expressing cells.

Fig. 19 is a graph showing the CTL activity of peptide-stimulated PBMC against NS5A-expressing cells.

Fig. 20 is a graph showing the specificity of the activity of anti-NS5A-2132 IgG.

Fig. 21 is a graph showing the results of a cell growth inhibition assay using anti-NS5A-2132 IgG.

Fig. 22 is a graph showing the results of assaying the ADCC activity of anti-NS5A-2132 IgG.

Fig. 23 is a graph showing the detection of an anti-C-35 antibody and an anti-NS5A-2132 antibody in patients with various diseases.

Fig. 24 is a graph showing the HLA or HCV genotype

restriction of anti-peptide antibodies.

Fig. 25 is a graph showing the HLA or HCV genotype restriction of anti-peptide antibodies.

Fig. 26 is a graph showing the results of measuring the level of anti-NS5A-2132 antibody in the sera of patients.

Fig. 27 is a graph showing the results of measuring the level of anti-NS5A-2132 IgG in the sera of patients.

Fig. 28 is a graph showing the levels of anti-NS5A antibody and HCV RNA in the sera of patients.

Fig. 29 is a graph showing the detection of an anti-C35 antibody and an anti-NS5A-2132 antibody in a cohort study.

Fig. 30 is a graph showing the detection of an anti-NS5A-2132 antibody in a cohort study.

Fig. 31 is a graph showing the results of measuring the IgG level in the blood of patients infected with HCV by a Luminex assay according to the present invention.

Fig. 32 is a graph showing the results of measuring the IgG level in the blood of patients infected with HCV by a Luminex assay according to the present invention.

Fig. 33 shows the course of a treatment of hepatitis C using peptides of the present invention.

Fig. 34 shows the course of a treatment of hepatitis C using peptides of the present invention.

Detailed Description of the Invention

The peptide derived from hepatitis C virus of the invention is an HCV peptide, which contains the HLA-binding motif in its sequence and is recognized by an antibody present in the blood of a patient infected with hepatitis C virus.

In addition, the HCV peptide according to the invention contains an HLA-binding motif, i.e., an HLA-A2- or HLA-A24-binding motif in its sequence. This HCV peptide is also characterized by having a high immunogenicity as well as being recognized by cellular and humoral immunity.

Examples of the HCV peptide include those peptides listed in Tables 1 and 3. One of the particularly preferred HCV peptides of the invention is a peptide having the HLA-A2-binding motif with the following amino acid sequence:

C-35: YLLPRRGPRL (SEQ ID NO: 1).
In a patient,
IgG antibody reactive with this peptide is detected with significant proportion in patients infected with HCV with the detection ratio of 93% and the specificity for HCV infection of 100%. Further, particularly preferred HCV peptides of the invention are peptides having the

HLA-A24-binding motif with any of the following amino
acid sequences:

NS5A-2132: RYAPACKPL (SEQ ID NO: 2);

E2-488: HYAPRPCGI (SEQ ID NO: 3);

E1-213: VYEAADMIM (SEQ ID NO: 4);

NS3-1081: VYHGAGSKTL (SEQ ID NO: 5);

C-176: IFLLALLSCL (SEQ ID NO: 6);

C-173: SFSIFLLALL (SEQ ID NO: 7);

EYVLLLFLL (SEQ ID NO: 8);

PYIEQGMQL (SEQ ID NO: 16);

IFTITKILL (SEQ ID NO: 20); and

SFAIKWEYVL (SEQ ID NO: 38).

In particular, NS5A-2132 is able to induce both cellular
and humoral immunity in many HLA-A24-positive patients.

The HCV peptide according to the invention may be
a peptide having a homology of at least 70%, preferably
80% or more, more preferably 90% or more, with the amino
acid sequence of any of the peptides listed above.

The HCV peptide according to the invention may
further comprise a peptide having a property of being
recognized by an HLA-A2- or HLA-A24-restricted cytotoxic
T cell (CTL). This type of antigenic peptide capable of
binding to HLA is generated by intracellular degradation
of an antigenic protein produced in a cell, and has a
sequence motif for each HLA type. A cytotoxic T cell

(CTL) recognizes a complex of the antigenic peptide and HLA and causes damage in an HCV-infected cell.

The polypeptide according to the invention comprises in its sequence the amino acid sequence of the peptide of the invention as described above, but the total number of amino acids is not particularly limited. In addition, in the polypeptide of the invention, the amino acid sequence other than that of the peptide are located at the N-terminal side and/or the C-terminal side or both sides of the amino acids of the peptide. Therefore, such a polypeptide has substantially the same function and effect as those of the above-mentioned peptide. When the term "peptide" is used herein, it should be construed that "polypeptide" is also included in the term unless otherwise specified.

The peptide having an amino acid sequence as described above can be obtained by a standard chemical synthesis, by an enzymatic degradation of a protein molecule, or by a recombinant DNA technique using a host transformed so as to express a nucleotide sequence encoding a desired amino acid sequence.

In the case where the desired peptide is produced by a chemical synthetic method, it can be produced by a technique which is well known per se and is commonly used in standard peptide chemistry. For example, the

peptide can be synthesized by a solid phase synthetic method with a peptide synthesizer. A crude peptide obtained in this way can be purified by any of the purification method commonly used in protein chemistry, such as salting out, ultrafiltration, reverse-phase chromatography, ion-exchange chromatography and affinity chromatography.

In the case where the desired peptide is produced by a recombinant DNA technique, the desired peptide can be obtained by, for example, integrating a DNA fragment encoding a desired amino acid sequence synthesized as described above into an appropriate expression vector, transforming a microorganism or an animal cell using this expression vector and then culturing the thus obtained transformant. The expression vector that may be used in the invention includes a plasmid, a viral vector, etc., which are well known in the art.

To transform a host cell with an expression vector in the peptide-producing technique described above, any of well known methods may be appropriately selected and used, such as a calcium chloride method, a calcium phosphate co-precipitation method, a DEAE-dextran method, a Lipofectin method or an electroporation method. The peptide can be purified by any of the above-mentioned purification methods from a cell extract or a culture

supernatant collected from the culture medium.

A nucleotide, which is one of the other aspects of the invention, includes an oligonucleotide or a polynucleotide encoding the above-mentioned peptide derived from hepatitis C virus or the above-mentioned polypeptide. The nucleotide may include a ribonucleotide and a deoxynucleotide. The nucleotide may be modified by a method known in the art. Examples of the modification of the nucleotide include labeling, methylation, "caps", substitution of one or more of naturally occurring nucleotides with an analog, and intranucleotide modification known in the art. Examples of the intranucleotide modification include nonionic modification (e.g., a methyl phosphonate, a phosphotriester, a phosphoramidate, etc.), ionic modification (e.g., a phosphorothioate, a phosphorodithioate etc.), modification of incorporating a pendant moiety such as a protein (e.g., a nuclease, a toxin, an antibody, a signal peptide etc.), modification with a chelating agent (e.g., a metal, boron etc.).

The above-mentioned nucleotide sequence allows for provision of a peptide or a polypeptide sequence of an HCV antigen encoded by the genome of HCV, as well as a peptide useful for a diagnostic test or as a component

of a vaccine.

Once the nucleotide of the invention is obtained, it becomes possible to construct a nucleotide probe and a peptide useful for diagnosing a disease associated with HCV infection or for screening HCV infection in the blood or a blood preparation. Based on this nucleotide sequence, a DNA oligomer having, for example, 24 to 30 nucleotides or even more may be synthesized. The nucleotide can also be used as a probe for detecting HCV RNA in the serum of a subject or for screening the presence of HCV in the blood or blood products.

Furthermore, the nucleotide sequence allows for design and production of an HCV-specific peptide useful as a reagent for testing the presence of an antibody against HCV. An antibody raised against a purified peptide having this sequence may be used for detecting an HCV antigen in a subject infected with HCV and in blood products which might have been prepared from the blood of a person infected with HCV.

In another aspects of the invention, the antibody includes a chimeric antibody, a modified antibody, a monovalent antibody, Fab, F(ab')$_2$, Fab', a single chain Fv (scFV) protein and a single domain antibody, which are reactive to the peptide derived from hepatitis C virus or the polypeptide. These antibodies can

immunologically recognize the peptide or the
polypeptide.

In another aspects of the invention, a vector is
a construct comprising the nucleotide and can transform
a selected host cell to express heterologous coding
sequences in the host. The expression vector may be
either a cloning vector or an integration-type vector.

The cloning vector may include, for example, a
plasmid and a virus (such as an adenovirus vector). The
cloning vector is a replicon that can transform a host
cell and has an ability to replicate in the cell (e.g.,
a plasmid, a chromosome, a virus, a cosmid and the like).

An integration vector is a vector that does not
serve as a replicon in a host cell, but has an ability
to integrate its contents into a replicon (typically a
chromosome) in the host to stably transform the host.

Another aspect of the invention provides a method
of inducing a cytotoxic T cell targeted to an HCV-infected
cell using the peptide. The induction method of the
invention may comprise, for example, contacting the
peptide with a cell expressing HLA-A2 to present the
peptide on the HLA-A2 molecule, stimulating a T cell with
the cell presenting the peptide with HLA-A2, and inducing
the T cell into CTL. The HLA-A2-expressing cell to be
used in this method may be collected from the blood of

an HCV patient, however, it can also be prepared by introducing a gene encoding HLA-A2 into a cell that does not express HLA-A2. Thus, the peptide according to the invention is useful for detection and diagnosis of HCV infection and is also useful as a pharmaceutical composition, such as a vaccine for the treatment of a disease associated with hepatitis C virus.

CTL as induced in this way will target and attack a cell infected with HCV, since a CTL may be also as a pharmaceutical composition for a cell therapy.

In another aspect of the invention, the peptide derived from hepatitis C virus, polypeptide, nucleotide and/or antibody or substance with an antibody-like activity is useful for detecting or diagnosing HCV infection.

The detection of HCV and the diagnosis of a disease associated with HCV can be carried out by, for example, detecting the presence of the peptide, detecting the presence or amount of the corresponding nucleotide sequence, determining the biological distribution of the peptide in an individual and/or determining the amount of the peptide present in a specimen derived from an individual. Such a method may utilize the interaction and/or reactivity with the nucleotide sequence encoding the peptide. In other words, the detection of HCV and

the diagnosis of a disease associated with HCV may be effected by assaying the peptide as a marker. The assay can be carried out by a well known method by, for example, an antigen-antibody reaction system, an enzymatic reaction system, and a PCR reaction system.

Monitoring of the effect of the treatment and the prediction the prognosis of a disease associated with HCV can be carried out by monitoring the concentration of an antibody reactive to the peptide in the blood of a subject having a disease associated with HCV. It is particularly preferred to measure the level of anti-C-35 IgG or anti-NS5A-2132 IgG in the blood of a subject.

In still another aspect of the invention, the pharmaceutical composition includes a vaccine. The vaccine comprises the peptide derived from hepatitis c virus, the polypeptide and/or the nucleotide, and may suitably contain a pharmaceutically acceptable adjuvant and/or carrier. An adjuvant can enhance the immune response, and may include incomplete Freund's adjuvant or aluminum hydroxide gel. The carrier may include, for example, a diluent such as PBS or distilled water, physiological saline.

The pharmaceutical composition of the invention can be administered orally, parenterally or transdermally depending on the administration form, for

example, via intravenous administration or subcutaneous administration. Examples of the dosage form include tablets, granules, soft capsules, hard capsules, liquids, oils, and emulsions. The dose of such a pharmaceutical composition can suitably selected depending on the symptoms of the patient to be treated. In general, the amount of the peptide may range from 0.1 to 10 mg per day for an adult, and the composition may be administered once every several days or several months.

The entire contents of all patents and reference documents explicitly cited in the present specification are incorporated herein by reference. In addition, the entire contents described in the specification and drawings of Japanese Patent Application No. 2003-330258 to which this application claims priority are also incorporated herein by reference.

Examples

The invention will be described in more detail with reference to Examples below. These Examples are provided to illustrate the invention more specifically, but does not limit the scope of the invention.

The abbreviations for amino acids used in the following Examples are as follows.

A denotes alanine, C, cysteine; D, aspartic acid;

E, glutamic acid; F, phenylalanine; G, glycine; H,
histidine; I, isoleucine; K, lysine; L, leucine; M,
methionine; P, proline; R, arginine; S, serine; T,
threonine; V, valine; and Y, tyrosine.

Example 1: Detection of IgG reactive to HCV peptide in
the sera of patients infected with HCV
Peptide

Synthetic peptides having an HLA-A2-binding motif
or an HLA-A24-binding motif from a conserved region of
HCV genotype 1b protein were used in this study (Table
1). A peptide derived from HIV having an HLA-A2-binding
motif was used as a negative control. All of these
peptides were purchased from a commercially supplier.
The purity was analyzed by reverse-phase high-pressure
liquid chromatography and found to be 90% or higher.

Table 1

No	Peptide name	alias	Region	Sequence	SEQ ID NO	HCV patients (n=12) Positive		Healthy donors (n = 10) Positive	
						n	%	n	%
3	1bA24-2132	NS5A-2132	NS5A	RYAPACKPL	2	12	100	1	10
4	1bA24-717	E2-717	E2	EYVLLLFLL	8	2	17	0	0
5	1bA24-1100	NS3-1100	NS3	MYTNVDQDL	9	0	0	0	0
6	1bA24-1773	NS4A-1773	NS4A	QYLAGLSTL	10	0	0	0	0
7	1bA24-790	E2-790	E2	LYGVWPLLL	11	0	0	0	0
8	1bA24-1031	NS3-1031	NS3	AYSQQTRGL	12	0	0	0	0
9	1bA24-834	NS2-834	NS2	HYKLFLARL	13	0	0	0	0
10	1bA24-1292	NS3-1292	NS3	TYATYGKFL	14	1	8	0	0
11	1bA24-1266	NS3-1266	NS3	AYMSKAHGI	15	6	50	0	0

12	1bA24-488	E2-488	E2	HYAPRPCGI	3	9	75	0	0
13	1bA24-213	E1-213	E1	VYEAADMIM	4	6	50	0	0
14	1bA24-1716	NS4B-1716	NS4B	PYIEQGMQL	16	1	8	0	0
15	1bA24-1767	NS4B-1767	NS4B	NFISGIQYL	17	0	0	0	0
16	1bA24-910	NS2-910	NS2	PYFVRAHGL	18	0	0	0	0
17	1bA24-1727	NS4A-1727	NS4A	QFKQKAIGL	19	3	25	0	0
18	1bA24-885	NS2-885	NS2	IFTITKILL	20	5	42	0	0
19	1bA24-135	C-135	C	GYIPIVGAPL	21	1	8	0	0
20	1bA24-631	E2-631	E2	MYVGGVEHRL	22	0	0	0	0
21	1bA24-932	NS2-932	NS2	HYVQMALMKL	23	5	42	0	0
22	1bA24-947	NS2-947	NS2	TYVYDHLTPL	24	7	58	0	0
23	1bA24-1854	NS4B-1854	NS4B	GYGAGVAGAL	25	2	17	0	0
24	1bA24-1243	NS3-1243	NS3	AYAAQGYKVL	26	2	17	0	0
25	1bA24-1081	NS3-1081	NS3	VYHGAGSKTL	5	6	50	0	0
26	1bA24-787	E2-787	E2	AYALYGVWPL	27	2	17	0	0
27	1bA24-617	E2-617	E2	HYPCTVNFTI	28	4	33	0	0
28	1bA24-1417	NS3-1417	NS3	YYRGLDVSVI	29	2	17	0	0
29	1bA24-2146	NS5A-2146	NS5A	TFLVGLNQYL	30	2	17	0	0
30	1bA24-822	NS2-822	NS2	VFVGLILLTL	31	4	33	0	0
31	1bA24-1556	NS3-1556	NS3	EFWESVFTGL	32	0	0	0	0
32	1bA24-176	C-176	C	IFLLALLSCL	6	7	58	0	0
33	1bA24-837	NS2-837	NS2	LFLARLIWWL	33	0	0	0	0
34	1bA24-848	NS2-848	NS2	YFITRAEAHL	34	1	8	1	10
35	1bA24-1792	NS4B-1792	NS4B	AFTASITSPL	35	0	0	0	0
36	1bA24-1990	NS5A-1990	NS5A	DFKTWLQSKL	36	1	8	0	0
37	1bA24-2121	NS5A-2121	NS5A	FFTEVDGVRL	37	1	8	0	0
38	1bA24-173	C-173	C	SFSIFLLALL	7	7	58	0	0
39	1bA24-711	E2-711	E2	SFAIKWEYVL	38	1	8	0	0
40	1bA2-35	C-35	C	YLLPRRGPRL	1	12	100	0	0
41	1bA2-132	C-132	C	DLMGYIPLV	39	0	0	0	0
42	1bA2-178	C-178	C	LLALLSCLTV	40	1	8	0	0
43	1bA2-220	E1-220	E1	ILHTPGCV	41	3	25	0	0
44	1bA2-363	E2-363	E2	SMVGNWAKV	42	0	0	0	0
45	1bA2-401	E2-401	(HVR)	SLLAPGAKQNV	43	0	0	3	30
46	1bA2-1073	NS3-1073	NS3	CINGVCWTV	44	0	0	0	0
47	1bA2-1169	NS3-1169	NS3	LLCPAGHAV	45	1	8	2	20
48	1bA2-1287	NS3-1287	NS3	KLVALGINAV	46	1	8	0	0
49	1bA2-1406	NS3-1406	NS3	TGAPVTYSTY	47	1	8	0	0
50	1bA2-1789	NS4B-1789	NS4B	SLMAFTAAV	48	0	0	0	0
51	1bA2-1807	NS4B-1807	NS4B	LLFNILGGWV	49	0	0	0	0
52	1bA2-1851	NS4B-1851	NS4B	ILAGYGAGV	50	0	0	1	10
53	1bA2-2252	NS5A-2252	NS5A	ILDSFDPLV	51		0	0	0
54	1bA2-2692	NS5B-2692	NS5B	RLIVFPDLGV	52	1	8	0	0
55	1bA2-2727	NS5B-2727	NS5B	GLQDCTMLV	53	2	17	1	10
56	1bA2-150	C-150	C	ALAHGVRAL	54	0	0	1	10
57	1bA2-168	C-168	C	NLPGCSFSI	55	0	0	1	10
60	1bA24-2422	NS5B-2422	NS5B	SYTWTGALI	56	0	0	0	0
61	1bA24-2482	NS5B-2482	NS5B	HYRDVLKEM	57	3	25	1	10
62	1bA24-2990	NS5B-2990	NS5B	WFMWCLLLL	58	7	58	0	0
63	1bA24-789	E2-789	E2	AFYGVWPLL	59	0	0	0	0
64	1bA24-1727	NS4B-1727	NS4B	QFKQKALGL	60	1	8	0	0
65	1bA24-2613	NS5B-2613	NS5B	QYSPGQRVEF	61	0	0	0	0

| 66 | 1bA24-1727 | NS4B-1727 | NS4B | QFKQKALGLL | 62 | 3 | 25 | 0 | 0 |

The cut-off value is 1.83.

Table 2

	No.	40
		prevalence
Diseases associated with HCV infection	60	56 (93.3%)
Subjects infected with hepatitis B virus	24	0 (0%)
Hepatitis B virus vaccine recipients	10	0 (0%)
Healthy donors	27	0 (0%)
Patients with an autoimmune disease	22	0 (0%)
Subjects infected with HTLV-I	10	8 (80%)
Subjects infected with HIV	3	3 (100%)
Total	156	67
		C-35
Sensitivity		93.3%
Sensitivity		88.5%
OD ratio		
		P<0.001

The detection rate was calculated based on the number
of positive patients with above the cutoff value (mean
± 3 SD).

The detection rates of NS-5A-2132 and C-35 are 0.202 and
0.13, respectively.

Statistical analysis was carried out by the χ^2-test.

ELISA

The level of the peptide-specific IgG in the serum

was measured by ELISA. First, each peptide was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. The peptide was diluted with 0.1 M sodium carbonate/sodium bicarbonate solution containing disuccinimidyl suberate (DSS) as a crosslinker. An ELISA plate was coated with 20 µg/well of peptide at 4°C overnight. The wells were washed 3 times with 0.05% Tween 20/PBS (PBST), and the plate was blocked with Block Ace (registered trademark) and left overnight at 4°C. Plasma or serum samples were diluted in 1:100, 1:200 and 1:400 with 0.05% Tween 20/Block Ace, and 100 µl of each of the samples was added to each well. After the samples were incubated at 37°C for 2 hours, the plate was washed 9 times with PBST, 100 µl of a 1:1000 diluted rabbit anti-human IgG (γ-chain specific) was added to each well, and incubated at 37°C for 2 hours. The plate was washed 9 times with PBST, and 100 µl of 1:100 diluted mouse anti-rabbit IgG conjugated to a horseradish peroxidase-dextran polymer was added to each well, and then the plate was incubated at room temperature for 40 minutes. After the plate was washed, 100 µl of a tetramethylbenzidine substrate solution was added to each well. The reaction was stopped by the addition of 2.0 M phosphoric acid. The cutoff value was calculated as the mean ± 3 SD of the optical density values of the control obtained from the healthy donors.

Absorption and elution of peptide-specific IgG

One hundred microliters of a plasma or serum sample
diluted with 0.05% Tween 20/Block Ace (registered
trademark) was added to each well and allowed to be
absorbed to the peptide (20 μg/well) coated on the well
of the plate at 37°C for 2 hours. After this procedure
was repeated 3 times, the peptide-specific IgG level in
the supernatant was measured by ELISA. The antibody
bound to the peptide-coated plate in the initial
adsorption step was eluted with 30 μl/well of 5 M NaCl/50
mM citrate buffer (pH 3.0) (neutralized with 0.05% Tween
20/Block Ace containing 1.0 M Tris-HCl (pH 7.5)), and
the peptide-specific IgG level in the eluted fraction
was measured by ELISA.

Assay for peptide-specific CTL

A peptide-specific CTL was detected by a method
commonly used in this technical field. PBMCs were added
to each well of a round-bottomed 96-well microculture
plate at 1×10^5 cells per well, and the cells were
incubated in 200 μl of a medium with 10 μM peptide. The
composition of this medium is as follows: 45% RPMI-1640,
45% AIM-V (registered trademark), 10% fetal calf serum
(FCS), 100 U/ml interleukin 2 (IL-2), and 1 mM MEM

non-essential amino acid solution. On days 3, 6 and 9 of culture, one half of each culture solution was removed and replaced with a fresh medium containing a corresponding peptide (20 μg/ml). On day 12 of culture, the cultured cells were collected and assayed for the interferon gamma (IFN-γ) production in response to CIR-A2402 cell or T2 previously pulsed with the corresponding peptide or an HIV peptide as a negative control. Four wells were used for each peptide and the assay was carried out twice. The background IFN-γ production in response to the HIV peptide was subtracted from the obtained data value. For the inhibition assay, a peptide-reactive CTL was purified using a CD8 isolation kit, and assayed for the peptide-specific IFN-γ production in the presence of 20 μg/ml of any of an anti-HLA class I (W6/32, IgG2a) monoclonal antibody, an anti-CD8 (Nu-Ts/c, IgG2a) monoclonal antibody and an anti-HLA class II (H-DR-1, IgG2a) monoclonal antibody. The background IFN-γ production was subtracted from the obtained data value.

Statistics

The values are expressed as the mean ± SD. A statistical analysis was carried out using the Mann-Whitney U-test and the chi-square test, and a P value

less than 0.05 was considered statistically significant.

Detection of antibody against HCV peptide

The level of IgG that reacts with each of the 62 types of peptides was measured in the sera from 12 patients infected with HCV and 10 healthy donors (HDs) (Table 1). The prevalence of the antibody against each peptide is shown in Table 1. A plurality of peptides were found that highly reacted with IgG in the blood from the patients infected with HCV and rarely react with the sera from the healthy donors.

The peptides include No. 3: NS5A-2132 (corresponding to the peptide at positions 2132-2140 of HCV NS5A protein, hereinafter abbreviated in a similar fashion), No. 11: NS3-1266, No. 12: E2-488, No. 13: E1-213, No. 22: NS2-947, No. 25: NS3-1081, No. 32: C-176, No. 38: C-173, No. 40: C-35, and No. 62: NS5B-2990. Representative results of measuring IgG level in the blood from the patients infected with HCV by the ELISA method are shown in Figs. 1 and 2.

In the serum from the patient shown in Fig. 1, an IgG antibody against C-35 was detected.

In the sera from 4 patients shown in Fig. 2 (A) to (D), IgG antibodies reactive to NS5A-2132 (A), NS3-1081 (B), E2-488 and E1-213 (C) and C-176 and C-173 (D) were

detected, respectively. The peptide specificity of those peptide-reactive antibodies was examined by absorption and elution tests.

Fig. 3 shows a representative example of the experimental results of absorption and elution of an antibody reactive to C-35 peptide in the serum of the patient shown in Fig. 1. This antibody was absorbed by C-35, but not by an irrelevant peptide NS5A-2252 (upper graph of Fig. 3). Furthermore, the antibody was eluted from the C-35-adsorbed fraction (the lower graph of Fig. 3).

Among the antibodies listed in Table 1, the antibody reactive to C-35 peptide was detected at 100% in the patients infected with HCV and at 0% in the healthy donors, indicating that it has high specificity to HCV. The sequence of C-35 peptide of HCV has a partial homology with peptides of various viruses, such as tet protein (the amino acid sequence at positions 54-58) or env protein (amino acid sequences at positions 5-9, 158-162, and 717-727) of HIV-1. It also shows a homology with various proteins of HTLV-I (such as pol 724-755, rex 5-9, rex 310-313 and tax 70-74). Thus, the antibody reactive to C-35 peptide was further examined in a double-blind study of 156 cases in total, including 60 cases of diseases associated with HCV infection (22 cases of

chronic hepatitis C, 20 cases of liver cirrhosis and 18

cases of liver cancer), 24 cases of subjects infected

with hepatitis B virus, 10 cases of hepatitis B virus

vaccine recipients, 27 cases of healthy donors, 22 cases

of patients with an autoimmune disease, 10 cases of

subjects infected with HTLV-I and 3 cases of subjects

infected with HIV. The results are shown in Table 2. The

HCV specificity and the detection rate of the antibody

reactive to C-35 peptide were 88.5% and 93.3%,

respectively.

Induction of peptide-specific CTL activity

Six types of HLA-A24 binding peptides (Nos. 3, 12,

13, 25, 32 and 38) and 18 types of HLA-A2 binding peptides

(Nos. 40 to 57) were incubated with peripheral blood

mononuclear cells (PBMCs) obtained from HLA-A24$^+$ or

HLA-A2$^+$ HCV-infected patients (5 patients in each group).

As a control, the PBMCs were incubated with an HIV peptide.

Then, the incubated PBMCs were tested for their ability

to produce IFN-γ in response to CIR-A2402 cell or T2 cell

pulsed with a corresponding peptide. Six types of

HLA-A24 binding peptides (Nos. 3, 12, 13, 25, 32 and 38)

could induce CTL from the PBMC obtained from 10 HLA-A24$^+$

HCV patients (Fig. 4). Furthermore, CTL could be induced

by the HLA-A2 binding peptides derived from HCV (Nos.

40 to 57) in the PBMC obtained from 5 HLA-A2$^+$ HCV patients

(Fig. 5).

The cytotoxic activity of peptide- stimulated PBMC

was measured by the ^{51}Cr release assay. The

representative results are shown in Figs. 6 and 7.

Fig. 6 shows the cytotoxic activity of CTL induced

in the PBMC obtained from the HLA-A24$^+$ HCV patients by

the HLA-A24 binding peptides (Nos. 3, 12, 13, 25, 32 and

38) derived from HCV. The cytotoxic activity against

C1R-A2402 cells loaded with the corresponding peptide

(represented by C1RA2402 in the drawing) was

significantly higher than that against the control

C1R-A2402 cells loaded with the HIV peptide (represented

by C1RA2402 HIV in the drawing).

Fig. 7 shows the cytotoxic activity of CTL induced

in the peripheral blood PBMC obtained from the HLA-A2$^+$

HCV patients by the HLA-A2 binding peptides (Nos. 40 and

41) derived from HCV. The cytotoxic activity against T2

cells loaded with the corresponding peptide (represented

by T2 in the drawing) was significantly higher than that

against the control T2 cells to loaded with the HIV

peptide (represented by T2HIV in the drawing).

These PBMCs showed a significantly higher level of

cytotoxic activity against the C1R-A2402 cell and the

T2 cell preloaded with the corresponding peptide (except

for an HIV peptide as a negative control).

The HLA class I restriction of the cytotoxicity was tested by the inhibition assay using an anti-CD8 monoclonal antibody (Fig. 8). Fig. 8 shows a representative example of inhibition of the CTL activity by the anti-CD8 antibody. The cytotoxicity of the CTL induced in the PBMC obtained from the HLA-A2$^+$ HCV patient by the HLA-A2 binding peptide (No. 40) derived from HCV was inhibited by the anti-CD8 antibody, but not by an anti-CD4 antibody or the control anti-CD14 antibody.

Example 2: Detection of IgG reactive to HCV peptide in the sera of HLA-A24$^+$ HCV-infected patients
Subject

The detection of IgG reactive to the HCV peptide was carried out more extensively in HLA-A24$^+$ HCV-infected patients. Sixty HCV-1b$^+$ patients were found to be seropositive for an anti-HCV antibody (Ab) indicated by the second generation or the third generation immunoassay test. The genotype of HCV contained in the sera of the patients was determined by RT-PCR. The patients were diagnosed at the time of serum sampling by biochemical analysis, echography, computed tomography and histological finding. The results of diagnosis are as follows: chronic hepatitis (CH, n = 24),

liver cirrhosis (LC, n = 18) and hepatic cell carcinoma
(HCC, n = 18). A negative control was serum samples from
20 healthy donors (HDs) who had normal hepatic function
and no medical history of hepatitis virus.

Assay of peptide and peptide-reactive antibody

Forty-four types of synthetic peptides that were
derived from a highly conserved region of HCV-1b protein
and selected based on a high binding score to HLA-A24
molecule (Bioinformatics and Molecular Analysis Section,
NIH, Bethesda, MD) were used. A peptide derived from HIV
that has an HLA-A24 binding motif (RYLRDQQLLGI (SEQ ID
NO: 63)) served as a negative control. The binding
scores and sequences of the peptides are shown in Table
3. For the screening of the reactivity of peptide to
serum IgG, desalted grade of the peptides (> 70%) were
purchased from BioSynthesis (Lewisville, TX) or SynPep
(Dublin, CA). A purified peptide (> 90%) was used in the
subsequent experiments. The plasma level of
peptide-specific IgG was measured by an enzyme-linked
immunosorbent assay (ELISA). The specificity of
anti-peptide IgG in a plasma sample was tested by the
absorption and elution of peptide-reactive IgG.

Assay of peptide-specific CTL

Peptide-specific CTL precursor cells were detected by the method as described in Hida et al. (Cancer Immunol Immunother 2002; 51: 219-228). For an inhibition assay, 20 µg/ml of anti-HLA class I (W6/32, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), anti-CD4 (Nu-Th/i, IgG1b) and anti-HLA class II (H-DR-1, IgG) monoclonal antibodies were used.

CTL activity of peptide-stimulated PBMC against cell transfected with NS5A gene

The cDNAs of NS5A, HLA-A2402 and HLA-A2601 were obtained by RT-PCR from Huh7 (NNU-50-1) cell, C1R-A2402 cell and KE4-CTL cell, respectively, and cloned into an expression vector pCR3.1 (Invitrogen, San Diego, CA). The Huh7 (NNU50-1) cell line is a replicon cell derived from a cell line infected with HCV-1b and expresses proteins from NS3 to NS5B (Kishine et al., Biochem Biophys Res Commun 2002; 293: 993-999). Then, 100 ng of NS5A, HLA-A2402 or NS5A and HLA-A2601 cDNA (as a negative control) were mixed in 100 µl of Opti-MEM (Invitrogen) containing 0.6 µl of Fugene6 (Roche Molecular Biochemicals, Indianapolis, IN) and incubated for 30 minutes. Then, 100 µl of the mixture was added to COS-7 cells (1 x 10^4 cells) and incubated for 6 hours. One hundred microliters of RPMI-1640 medium containing 20% FCS was added to the mixture and COS-7 cells were cultured

for 2 days, followed by the addition of NS5A

2132-2140-stimulated PBMCs (2 x 10^5 cells/well). After

incubation for 18 hours, 100 µl of the supernatant was

removed and IFN-γ concentration was assayed by ELISA in

triplicate. In order to prepare a stable transfectant

cell line, either pCR3.1-NS5A or pCR3.1-HLA-A3101

(negative control) was transfected to C1R-A2402 cell by

electroporation using a Gene Pulser (BioRAD, Richmond,

CA). These cells were cultured for 30 to 40 days in the

presence of geneticin (G418), and then geneticin

resistant cells were collected. The expression of NS5A

protein in the transfectant was assayed by Western blot

analysis using an anti-NS5 polyclonal antibody (Abcam

Limited, Cambridge, UK).

Growth inhibition and antibody dependent cellular
cytotoxicity (ADCC)

Huh7 cell line was incubated in a well of a

flat-bottomed 96-well microculture plate in the presence

of various sera for 24, 48 and 72 hours. After the

incubation, Huh7 cells were counted with a Cell Count

kit-8 (10 µl/well) (Dojindo, Japan). For ADCC assay,

PBMCs newly isolated from a HLA-A24$^+$ HD were

pre-incubated for 1.5 hours in 10% FCS and RPMI-1640

medium containing inactivated serum (56°C, 30 minutes)

from any of various patients and HD (as a negative
control). C1R-A2402 cells or C1R cells (as a negative
control) were incubated for 2 hours with NS5A 2132-2140
peptide (10 μM) and radiolabeled with $Na_2{}^{51}CrO_4$ for 1.5
hours, washed and used as target cells. The
peptide-stimulated PBMCs were incubated in a well of a
round-bottomed 96-well microculture plate with the
target cells at an effecter to the target cell (E/T) ratio
of 40, 20 or 10 to 1. After incubation at 37°C for 6 hours,
cell-free supernatant was collected and the ADCC
activity was assayed (Shomura, et al., Br J Cancer 2004;
90: 1563-1571).

Statistics

A statistical analysis was carried out using
Student's t-test and the Mann-Whitney U-test. Values of
P < 0.05 were considered statistically significant.

Detection of humoral response to HCV-peptide

In order to screen for a humoral response to the
peptides, the level of IgG reactive to each of the 44
types of peptides was assayed in the plasma of 12 patients
infected with HCV-1b. The plasma of 10 HDs was used as
a negative control. The level of peptide-reactive IgG
was measured by ELISA for serially diluted plasma samples

and reported by the values of absorbance (OD) of each sample. The cut-off value of these peptides at a plasma dilution of 1:100 was set at 0.18 OD (mean (0.08) + 2SD (0.05 x 2)of 10 HDs). A significant level (> 0.18 OD at a plasma dilution of 1:100) of IgG reactive to NS5A-2132, C-176, E2-488, E1-213, C-173 and NS3-1081 peptides were detectable in the plasma of 12, 11, 10, 9, 8 and 5 patients among the 12 patients, respectively (Fig. 9). As expected, none of these peptides was reactive to the plasma from the 10 HDs. The reactivity of the peptides to peptide-specific IgG is summarized in Table 3.

Table 3: HCV restricted peptides and reactivity to peptide-specific IgG [a]

No.	Region	Peptide	SEQ ID NO	Reactivity to peptide-specific IgG		
				Binding score [b]	Patients (n = 12)	HD (n = 10)
1	C 135-144	GYGAGVAGAL	25	504	1	0
2	C 173-182	SFSIFLLALL	7	24	8	0
3	C 176-185	IFLLALLSCL	6	36	11	0
4	E1 213-221	VYEAADMIM	4	37.5	9	0
5	E2 488-496	HYAPRPCGI	3	60	10	0
6	E2 617-626	HYPCTVNFTI	28	75	2	0
7	E2 631-640	MYVGGVEHRL	22	420	0	0
8	E2 711-720	SFAIKWEYVL	38	20	1	0
9	E2 717-725	EYVLLLFLL	8	432	2	0
10	E2 787-796	AYALYGVWPL	27	200	2	0
11	E2 789-797	AFYGVWPLL	59	20	0	0
12	E2 790-798	LYGVWPLLL	11	200	0	0

13	NS2 822-831	VFVGLILLTL	31	30	2	0
14	NS2 834-842	HYKLFLARL	13	60	0	0
15	NS2 837-846	LFLARLIWWL	33	36	0	0
16	NS2 848-856	YFITREAHL	34	30	1	1
17	NS2 885-893	IFTITKILL	20	20	3	0
18	NS2 910-918	PYFVRAHGL	18	24	0	0
19	NS2 932-941	HYVQMALMKL	23	396	3	0
20	NS2 947-956	TYVYDHLTPL	24	300	2	0
21	NS3 1031-1039	AYSQQTRGL	12	200	0	0
22	NS3 1081-1090	VYHGAGSKTL	5	200	5	0
23	NS3 1100-1108	MYTNVDQDL	9	336	0	0
24	NS3 1243-1252	AYAAQGYKVL	26	200	2	0
25	NS3 1266-1274	AYMSKAHGI	15	75	2	0
26	NS3 1292-1300	TYSTYGKFL	14	200	1	0
27	NS3 1417-1426	YYRGLDVSVI	29	50	2	0
28	NS3 1556-1565	EFWESVFTGL	32	40.32	0	0
29	NS4B 1716-1724	PYIEQGMQL	16	36	1	0
30	NS4B 1727-1735	QFKQKAIGL	19	20	3	0
31	NS4B 1727-1735	QFKQKALGL	60	20	1	0
32	NS4B 1727-1736	QFKQKALGLL	62	20	1	0
33	NS4B 1767-1775	NFISGIQYL	17	36	0	0
34	NS4B 1773-1781	QYLAGLSTL	10	300	0	0
35	NS4B 1792-1801	AFTASITSPL	35	28	0	0
36	NS4B 1854-1863	GYGAGVAGAL	25	280	2	0
37	NS5A 1990-1999	DFKTWLQSKL	36	26.4	1	0
38	NS5A 2121-2130	FFTEVDGVRL	37	24	1	0
39	NS5A 2132-2140	RYAPACKPL	2	480	12	1
40	NS5A 2146-2156	TFLVGNQYL	30	43.2	2	0
41	NS5B 2442-2451	SYTWTGALI	56	50	0	0
42	NS5B 2482-2491	HYRDVLKEM	57	46.2	3	1
43	NS5B 2613-2622	QYSPGQRVEF	61	132	0	0
44	NS5B 2990-2998	WFMWCLLLL	58	30	3	0

a) The level of peptide-specific IgG in the sera of 12 HCV-1b^{+} patients was measured by ELISA.

The cut-off value was determined to be 0.18.

b) The binging score of the peptide to HLA-A24 molecule was searched using Bioinformatics and Molecular Analysis Section, NIH, Bethesda, MD (http://bimas.dcrt.nih.gov/molbio/hla_bind/).

Each of the 6 types of the above peptides having

a purity of 90% or more and the negative control peptide were tested for reactivity to each of the serum samples from 60 HCV-1b⁺ patients (CH, n = 24; LC, n = 18; and HCC, n = 18) and 20 HDs (Fig. 10).

The values for each of these samples at a dilution of 100:1 were plotted. The mean ± SD of IgG (OD value) was as follows: NS5A-2132 (0.48 ± 0.36), E2-488 (0.18 ± 0.19), E1-213 (0.10 ± 0.21), NS3-1081 (0.036 ± 0.14), C-176 (0.09 ± 0.14) and C-173 (0.04 ± 0.13). The asterisks indicate P < 0.05 in the Mann-Whitney U-test. The average level of IgG reactive to NS5A-2132, E2-488, E1-213 or C-173 was statistically significantly higher than that of HDs, however, it was not the case with NS3-1081 or C-176. IgG showing a significant level of reactivity (> 0.101 OD at a serum dilution of 1:100) to NS5A-2132, E2-488, E1-213, C-173, NS3-1081 and C-176 peptides was detected in the plasma of 57 (95%), 44 (73%), 28 (47%), 23 (38%), 21 (35%) and 17 (28%) patients of the 60 patients tested, respectively. On the contrary, a significant level of IgG could not be detected in any of the sera of 20 HDs (Fig. 10).

The peptide specificity of IgG reactive to each of these peptides was examined by absorption and elution tests. Each serum sample was absorbed by either a corresponding peptide or an irrelevant peptide (HIV;

used as a negative control) at 37°C for 3 times and the level of peptide-specific IgG was measured by ELISA (Fig. 11). In the elution test, IgG molecules bound to a peptide- immobilized plate was eluted with either a corresponding peptide or an irrelevant peptide after the first absorption, and then the level of peptide-specific IgG in an eluted fraction was measured by ELISA. Representative results of the mean ± SD of OD values from three measurements is shown in the figure (Fig. 12). The asterisks indicate $p < 0.05$ in the two-sided Student's t-test.

As expected, each IgG to the 6 types of peptides was all absorbed by the corresponding peptide, but not absorbed by the irrelevant peptide (HIV peptide). Furthermore, this IgG was eluted from a binging fraction by the corresponding peptide, but not eluted by the irrelevant peptide. Taken together, these results suggested that IgG reactive to each peptide be specific to the peptide derived from HCV-1b.

Induction of peptide-specific cellular response

PBMCs from HLA-A24[+] patients infected with HCV-1b (n = 12, 6 CH patients, 3 LC patients, and 3 HCC patients) and HLA-A24[+] HDs (n = 5) without HCV infection were cultured with each of the 6 types of peptides (purity

> 90%) or a control HIV peptide for 13 days, and examined

for IFN production in response to C1R-A2402 cell pulsed

with a corresponding peptide. PBMCs from HLA-A24$^+$

HCV-1b$^+$ patients (n = 12) and HDs (n = 5) were stimulated

with any of the 6 types of peptides in 4 wells (15 x

10^4/well). On day 14 of the culture, the

peptide-stimulated PBMCs (80 to 120 x 10^4/well) were

separately collected from each well and divided into

quarters. Two portions of them were tested separately

for the ability to produce IFN-γ in response to C1R-A2402

cells pulsed with the corresponding peptide. The

remaining two portions were tested with cells using the

negative control peptide (HIV). The level of IFN-γ

produced in response to the HIV peptide (< 50 pg/ml) was

subtracted as background. The asterisks indicate p <

0.05 in the two-sided Student's t-test.

HCV peptides of C-173, C-176, E1-213, E2-488,

NS3-1081 and NS5A-2132 induced IFN-γ production at a

significant level (p < 0.05) in 1, 3, 4, 6, 2 and 7 of

12 patients (Figs. 13 and 14) and in 0, 0, 1, 1, 1, and

1 of 5 HDs (data not shown), respectively. Among these

6 types of peptides, NS5A-2132 peptide was recognized

by cellular immunity and humoral immunity in PBMCs from

7 of 12 patients and in the sera of 57 of 60 HCV-1b$^+$

patients tested.

The cytotoxicity of these peptide-stimulated PBMC was examined by a 6-hour ^{51}Cr release assay (Fig. 15). Peptide-stimulated PBMC showing positive response in the IFN-γ production assay (described above) was cultured and grown in vitro only with IL-2. Then, cytotoxicity against C1R-A2402 cell pulsed with a corresponding peptide or an HIV peptide (negative control) was tested by a standard 6-hour ^{51}Cr release assay at three different E/T cell ratios. Representative results are shown in the figure. The values are expressed as the mean ± SD of percentage of specific lysis. The asterisks indicate p < 0.05 in the two-sided Student's t-test. The PBMC stimulated by NS5A-2132, E2-488, or E1-213 peptide showed a significant level of cytotoxicity against C1R-A2402 cell pre-loaded with a corresponding peptide but not with the HIV peptide (Fig. 15).

The peptide-stimulated PBMC used in the experiment shown in Fig. 15 was tested for cytotoxicity against C1R-A2402 cells pulsed with a corresponding peptide in the presence of 20 μg/ml of an anti-HLA-class I (W6/32, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a) or anti-CD4 (Nu-Th/i, IgG1) monoclonal antibody (mAb). An anti-CD14 (JML-H14, IgG2a) mAb was used as a negative control. A 6-hour ^{51}Cr release assay was carried out at three different E/T ratios. The

values are expressed as the mean ± SD of percentage of specific cytotoxicity (Fig. 16). The asterisks indicate $P < 0.05$ in the two-sided Student's t-test.

Cytotoxicity against C1R-A2402 cells pulsed with each of 3 types of peptides was inhibited by the anti-HLA-class I (W6/32) or CD8 monoclonal antibody (mAb), but not by any of the other mAbs tested, indicating that the peptide-specific cytotoxicity is mediated mainly by $CD8^+T$ cells in an HLA-class I restricted manner (Fig. 16). On the contrary, such cytotoxicity was not detectable in the PBMCs stimulated with the remaining 3 types of peptides (C-173, C-176 and NS3-1081) (data not shown).

Then, it was examined whether NS5A-2132 peptide-stimulated PBMCs recognize a naturally processed peptide using COS-7 cell that was transiently co-transfected with NS5A and HLA-A2401 genes as a target cell. As a negative control, COS-7 cell that was transiently co-transfected with NS5A and HLA-A2601 genes was used. The NS5A-2132 peptide-stimulated PBMCs from patients #1 and #2 were tested for their ability to recognize COS-7 cell transiently co-transfected with NS5A and HLA-A2401 genes as a target cell to produce IFN-γ. As a negative control, COS-7 cell that was transiently co-transfected with NS5A and HLA-A2601 genes was used.

The values are expressed as the mean ± SD of percentage
of IFN-γ production in triplicate assays at an E/T ratio
of 20:1 (Fig. 17). The asterisks indicate $p < 0.05$ in
the two-sided Student's t-test.

As expected, the peptide-stimulated PBMCs from
patients #1 and #2 showing a CTL activity (Fig. 13)
recognized COS-7 cell co-transfected with NS5A and
HLA-A2401 genes and produced a significant amount of
IFN-γ (Fig. 17). On the contrary, these PBMCs did not
react with COS-7 cell having NS5A and HLA-A2601 genes
as a negative control. In addition, the
peptide-stimulated PBMCs from other two patients (#3 and
#4) who did not show a CTL activity failed to produce
a significant amount of IFN-γ by the recognition of COS-7
cell co-transfected with NS5A and HLA-A2401 genes (data
not shown).

Next, cytotoxicity was tested against C1R-A2402
cell that was stably transfected with NS5A gene and used
as a target cell. The expression of HLA-A24 molecule in
C1R-A2402 cell that was stably transfected with NS5A gene
and the expression of HLA-A31 molecule in C1R-A2402 cell
that was stably transfected with HLA-A31 gene (negative
control) were analyzed by a flow cytometry using a FACScan
(Fig. 18).

Then, cytotoxicity was tested by a 6-hour ^{51}Cr

release assay using C1R-A2402 cell that was stably transfected with NS5A gene as a target cell. C1R-A2402 cell that was stably transfected with a negative control gene (HLA-A3101) was used as a negative control, and C1R-A2402 cell that was pulsed with NS5A-2132 peptide was used as a positive control. A 6-hour ^{51}Cr release assay was carried out at three different E/T ratios. The values are expressed as the mean ± SD of percentage of specific lysis. The asterisks indicate $p < 0.05$ in the two-sided Student's t-test. The PBMC stimulated with NS5A-2132 peptide showed a higher cytotoxicity level against both C1R-A2402 cell that was transfected with NS5A gene and non-transfected C1R-A2402 cell that was previously pulsed with a corresponding peptide, compared with the cytotoxicity against C1R-A2402 cell that was transfected with the negative control gene (Fig. 19). These results suggest that NS5A-2132 peptide-stimulated PBMC successfully recognized a peptide processed and produced naturally on HLA-A2402 molecule of HCV-1b$^+$ cell.

Further examination on anti-NS5A-2132 IgG

In order to understand more about the possibility of biological role of anti-peptide IgG, it was examined whether anti-NS5A-2132 IgG recognizes the whole NS5A protein in absorption and elution assays. NS5A-2132 and

an HIV peptide were used as a positive control and a negative control, respectively (see the description above for the details of the absorption and elution tests). Anti-peptide IgG was neither absorbed nor eluted with the whole NS5 protein (Fig. 20), suggesting that this peptide IgG did not react with the whole NS5 protein.

Then, Huh7 cells were incubated up to three days in the presence of sera from patients (sera from 2 HCV-1b$^+$ patients whose sera showed a high level of anti-NS5A-2132 activity). As a negative control, sera from 2 HDs and FCS were used. The number of Huh7 (NNU50-1) cells that could survive was counted with a Cell Count kit-8 (10 µl/well) and the mean value of three assays are shown. None of the tested sera inhibited the growth of Huh7 (NNU50-1) cells under these culture conditions (Fig. 21). Also it was examined whether anti-NS5A-2132 IgG has the ability to mediate ADCC activity. PBMC newly isolated from an HLA-A24$^+$ HD was tested for cytotoxicity against C1R-2402 cell previously pulsed with this peptide in the presence of the above-mentioned 4 types of inactivated sera. However, none of the sera tested showed ADCC activity under these conditions (Fig. 22).

Example 3: Prediction of prognosis of patient infected with HCV

Subjects

Sera were obtained from 33 patients with a disease associated with HCV in the cohort study conducted between 1995 and 2002. The results of the follow-up survey on the 33 patients are shown in Table 4. The sera obtained from patients with chronic hepatitis (CH, n = 68), liver cirrhosis (LC, n = 43) and hepatic cell carcinoma (HCC, n = 52) were also used in the analysis. These patients were diagnosed at the time of the first serum sampling by biochemical and histological findings, echography and computed tomography. An anti-HCV antibody was assayed using a chemiluminescence enzyme immunoassay (CLEIA) kit (Lumipulse II HCV, Fujirebio Inc., Tokyo, Japan) or the second generation or the third generation enzyme immunoassay test (SRL, Tokyo, Japan). HCV-RNA in the sera was detected using RT-PCR (SRL, Tokyo, Japan). The HCV genotype was determined by directly sequencing HCV in the sera of the patients by RT-PCR (SRL, Tokyo, Japan). The sera were also obtained from subjects who were not infected with HCV, which include 24 subjects with an autoimmune disease (6 patients with systematic lupus erythematosus, one with Behcet's disease and 17 with atopic dermatitis), 17 cases of hepatitis B virus (HBV) infection (positive for HBV surface antigen), 3 patients having human immunodeficiency virus (HIV) and 10 cases

with human T cell leukemia virus type I (HTLV-1) infection.
As a negative control, the sera obtained from 37 persons
who did not have the history of a viral hepatitis or
vaccination of HBV and had normal hepatic function were
tested.

Table 4: Diagnosis results in 1995 and 2002

1995	n	2002	n	Sex (M/F)	Age ± SD
CH	17	CH	12	3/9	67.7 ± 11.9
		LC	3	2/1	59.0 ± 7.9
		CH; with HCC	1	-/1	82
		LC; with HCC	1	1/-	65
LC	1	LC	1	-/1	69
ASC	4	CH	1	-/1	75
		ASC	1	-/1	76
		Past medical history of HCV infection	2	-/2	57.7 ± 6.36
Past medical history of HCV infection	11	Past medical history of HCV infection	11	2/9	68.8 ± 10.71
Total	33		33	8/25	67.1 ± 10.6

CH: chromic hepatitis; LC: liver cirrhosis;
HCC: hepatic cell carcinoma;
ASC: asymptomatic healthy carrier

Peptide

Two peptides with a purity of 90% or more were
purchased from BIOSYNTHESIS (Lewisville, TX): HCV-1b
core protein 35-44 (YLLPRRGPRL (SEQ ID NO: 1) capable
of inducing an HLA-A2-restricted CTL activity) and
HCV-1b NS5A protein 2132-2140 (RYAPACKPL (SEQ ID NO: 2)

capable of inducing an HLA-A24-restricted CTL activity).
As a negative control, peptides derived from HIV with
an HLA-A2-binding motif (SLYNTVATL (SEQ ID NO: 64)) and
an HLA-A24-binding motif (RYLRDQQLLGI (SEQ ID NO: 63))
were used.

Assay of antibody reactive to peptide

The level of peptide-specific IgG was measured by
an enzyme-linked immunosorbent assay (ELISA). Briefly,
each peptide was dissolved in dimethyl sulfoxide (DMSO)
and stored at -20°C. The peptide (20 μg/well) diluted
with 0.1 M carbonate buffer containing disuccinimidyl
suberate (DSS) (PIERCE, Rockford, IL) as a chemical
crosslinker was bound to an ELISA plate. The wells were
washed 3 times with 0.05% Tween 20/PBS (PBST). Then, the
plate was blocked overnight at 4°C with Block Ace
(Yukijirushi, Tokyo, Japan). A serum sample was diluted
1:100, 1:200 or 1:400 with 0.05% Tween 20/Block Ace, and
100 μl/well of the sample was added to each well. After
the sample was incubated at 37°C for 2 hours, the plate
was washed 9 times with PBST, and further incubated at
37°C for 2 hours with 100 μl/well of a 1:1000 diluted
rabbit anti-human IgG (γ-chain specific) (DAKO Glostrup,
Denmark). After the plate was washed 9 times, 100
μl/well of a 1:100 diluted anti-rabbit IgG-conjugated

horseradish peroxidase- dextran polymer (En Vision, DAKO) was added to each well, and then the plate was incubated at room temperature for 40 minutes. After the plate was washed, 100 µl/well of a tetramethylbenzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1.0 M phosphoric acid.

Statistics

A statistical analysis was carried out using the χ^2-test. Values of $P < 0.05$ were considered statistically significant.

Cross reactivity, HLA restriction and genotype restriction

The cross reactivity of two antibodies reactive to the peptides derived from HCV-1b was examined. The sera obtained from 60 HCV patients and patients who are not included in the cohort study (CH, n = 22, LC, n = 21 and HCC, n = 17) were examined for reactivity to the peptides of the core 35-44 (C-35) and NS5A 2132-2140 (NS5A-2132). The sera were also obtained from 24 subjects with an autoimmune disease, 17 cases of HBV infection and 10 cases of HTLV-1 infection. The sera obtained from 37 HDs were used as a negative control. The level of

peptide-reactive IgG contained in the serially diluted serum samples was measured by ELISA. The results are reported by the absorbance (OD) of each sample. The representative results of OD at a serum dilution of 100:1 are shown. The cut-off value was set at 0.093 (mean + 2 SD of OD from HDs). The statistical analysis was carried out using the χ^2-test. Values of P < 0.05 were considered statistically significant.

As a result, a significant level of anti-C-35 IgG was detected in 56 out of 60 HCV-positive patients (93.3%) and there was no significant difference in the IgG level among the 3 groups (CH, LC and HCC patients) (Fig. 23). Except for the HTLV-1 patients, the sera from different groups were not positive for the anti-C-35 antibody. In the sera of 8 out of 10 cases of HTLV-1$^+$ subjects, a low but significant level of anti-C-35 IgG was detected. A significant level of anti-NS5A-2132 IgG was detected in 45 out of 60 patients (75%). The IgG level was high in the CH patients, intermediate in the LC patients and low in the HCC patients in the 3 groups (p < 0.05 vs CH) (Fig. 23). The sera from other groups were positive for anti-NS5A-2132 IgG in the majority of tested cases. Further, the sera of 3 out of 37 HDs were positive for anti-NS5A-2132 IgG.

Then, the correlation between the HLA-class IA

phenotype and the level of anti-C-35 IgG or
anti-NS5A-2132 IgG was examined in 29 patients with a
disease associated with HCV. The HLA-class IA phenotype
was determined by a standard serological method. Nine
patients were HLA-A2$^+$, 13 patients were A24$^+$, and the
remaining 7 patients were A2$^-$A24$^-$. The levels of
Anti-C-35 and anti-NS5A-2132 were measured by a standard
ELISA and the OD value of each patient at a serum dilution
of 100:1 is shown in the figure. Regardless of
difference in the HLA-class IA phenotype, both
antibodies were detected in the majority of the patients
infected with HCV (Fig. 24).

Also the correlation between the HCV genotype and
the level of anti-C-35 IgG or anti-NS5A-2132 IgG was
examined in a double blind study in patients infected
with HCV-1b (n = 29), HCV-2a (n = 16) and HCV-2b (n =
3). The HCV genotype was determined by directly
sequencing HCV in the serum of the patient. The results
of all subjects at a dilution of 100:1 are shown in Fig.
25. The levels of anti-C-35 and anti-NS5A-2132 were
measured by a standard ELISA and the OD value of each
patient at a serum dilution of 100:1 is shown. The
cut-off value was set at 0.093 (mean + 2 SD of OD from
HDs).

The anti-C-35 antibody was detected in 26 out of

30 patients infected with HCV-1b, 15 out of 15 patients infected with HCV-2a and 3 out of 3 patients infected with HCV-2b, respectively. Similarly, the anti-NS5A-2132 antibody was detected in the sera of 23 out of 30 patients infected with HCV-1b, 10 out of 16 patients infected with HCV-2a and 3 out of 3 patients infected with HCV-2b, respectively (Fig. 25). These results indicate that both anti-C-35 antibody and anti-NS5A-2132 antibody were detected in the HCV-positive patients regardless of difference in the HLA-class IA subtype and in the HCV genotype.

The above results suggest that the level of the anti-NS5A-2132 antibody correlates with the prognosis of an individual infected with HCV but not the case with the anti-C-35 antibody.

Then, the sera of CH (n = 24), LC (n = 22), HCC (n = 26) and HDs (n = 9) were newly prepared and the level of anti-NS5A-2132 IgG was measured (Fig. 26). The values of mean ± SD for the CH, LC, HCC and HD groups were 0.51 ± 0.24, 0.27 ± 0.20, 0.26 ± 0.23 and 0.08 ± 0.07, respectively. The levels of the anti-NS5A-2132 antibody in the LC and HCC patients were significantly lower than that in the CH patients ($p < 0.05$), but still higher than that in the HDs. In order to compare these results with the results measured by a standard third generation assay,

the level of anti-HCV antibody was measured for all the
sera using a commercially available kit (Third
generation assay, SRL). The level is represented by an
exponent (the left column). The correlation between the
anti-HCV level and the anti-NS5A- 2132 level represented
by an exponent is shown in the right column. The levels
of the anti-HCV antibody measured using the third
generation assay were not significantly different among
the 3 groups (CH: 10 ± 2.7, LC: 11 ± 1.4, HCC: 11 ± 1.1)
(Fig. 27). Further, the HCV RNA level was measured for
these sera using a commercially available kit (SRL,
Japan). The HCV RNA level of the HCC patients (360 ± 269.6) was lower than that of the CH patients (610 ± 347.3)
or the LC patients (610 ± 246.4) $(p < 0.05)$ (Fig. 28).
However, there was no apparent correlation between the
level of the anti-NS5A antibody and the HCV RNA level
(Fig. 28).

Result of cohort study

In order to further examine the correlation between
the anti-NS5A-2132 antibody and the prognosis of an
HCV-positive patient at an individual level, the serum
levels of the two antibodies were examined in a sample
from the cohort study, in which habitants infected with
HCV were annually screened from 1990 to 2002. This study

was done with 66 serum samples in total, which were
obtained from 33 patients and collected twice in 1995
and 2002 from the same individuals. The diagnoses of
these 33 patients were CH (n = 17), LC (n = 1),
asymptomatic carrier (ASC) (n = 4), and a past medical
history of HCV infection (an individual spontaneously
recovered) (n = 11) at the time of 1995, and CH (n = 13),
LC (n = 4), HCC (n = 2), asymptomatic carrier (ASC) (n
= 1), and a past medical history of HCV infection (n =
13) at the time of 2002. In other words, in the period
of seven years, the progress of disease was not observed
in 26 patients, while the progress was observed in the
remaining 7 patients (Table 4). The OD values of the
anti-C-35 antibody and the anti-NS5A-2132 IgG in each
patient measured at a serum dilution of 100:1 in 1995
and 2002 are shown in Fig. 29. As expected, the levels
of the anti-C-35 antibody measured in 1995 and 2002 were
almost equal regardless of the conditions of the disease
in the majority of the 33 cases. On the contrary, the
level of the anti-NS5A antibody measured in 1995
decreased when measured in 2002 in all the 7 patients
whose diseases had progressed, whereas the values were
almost equal to those measured in 2002 in the majority
of the 25 cases in which the diseases had not progressed.
The median value ± SD of the anti-NS5A-2132 antibody in

the sera of the 7 patients whose diseases had progressed measured at the time of 1995 (0.67 ± 0.13) was significantly higher than the value (0.27 ± 0.11) measured in 2002 (p < 0.05).

Then, the 33 subjects were subdivided into 5 groups (CH, LC, HCC, a past medical history of HCV infection (a recovered individual), and ASC) and the levels of the two antibodies at a serum dilution of 100:1 in 2002 were plotted (Fig. 30). A statistical analysis was carried out using the χ^2-test. Values of P < 0.05 were considered to be statistically significant. The level of the anti-C-35 antibody was high in any of CH, LC, and HCC patients, and it became very low or undetectable in the recovered individuals. On the other hand, the level of the anti-NS5A-2132 antibody was high in CH, the recovered individual and ASC, intermediate in LC and the lowest in the HCC patients (p < 0.05 vs CH).

Example 4: Detection of IgG reactive to HCV peptide in the sera of HLA-A24$^+$ HCV-infected patients using Luminex assay

The level of IgG reactive to HCV peptide in the sera of HLA-A24$^+$ HCV-infected patients was measured in the same manner as in Example 1 using the Luminex assay, which is considered to be more sensitive than the ELISA method.

Coupling of peptide to microbeads

Each peptide was coupled to microbeads
(manufactured by Luminex Corporation, xMAP
Multi-Analyte COOH Beads), which was encoded with a
classification code for the content of each fluorescent
dye (hereinafter referred to as color-coded) according
to the manufacturer's instruction. One hundred
microliters of unprepared color-coded microbeads were
put in each well of a filter plate and aspirated, and
then washed twice with a washing buffer (phosphate
buffered saline (PBS) (pH 7.4 ± 0.1), Tween (registered
trademark) 20 (0.05% v/v)) and each well was aspirated
at the same time. Then, 50 μl of 0.1 M MES
(2-morpholinoethanesulfonic acid) buffer (pH 7.0) was
added, and 10 μl of EDC (N-ethyl-N'-(3-
dimethylaminopropyl)-carbodiimide hydrochloride) (1
mg/ml/0.1 M MES buffer (pH 7.0)) was added to each well.
Several hundred microliters of the peptide (1 mg/ml, 0.1
M MES buffer, pH 7.0) was mixed with the washed microbeads
in each well. After the peptide and the mixed microbeads
were reacted in the dark for 20 minutes at room
temperature, 10 μl of EDC (1 mg/ml/0.1 M MES buffer (pH
7.0)) was added to each well and the mixture was allowed
to react in the dark for 20 minutes at room temperature.

this process was repeated twice. After the excess
solution was aspirated, 100 μl of 1 M Tris-HCl buffer
(pH 7.0) was added to each well and the mixture was allowed
to react in the dark for 15 minutes at room temperature.
Then, the beads in each well were washed 3 times with
the washing buffer as above and collected in a storage
solution containing 0.05% sodium azide in Block Ace
(registered trademark).

Preparation of microbead mixture

Microbeads for each well are prepared by placing
a bead solution obtained by binding a peptide to the
color-coded microbeads in such a manner that about 5,000
microbeads are contained in each well of a filter plate
(each well contains about 1 μl of single type of bead).
A bead mixture was prepared by mixing equal amount of
ten types of microbeads prepared in this manner and
diluting the mixture with the above-mentioned washing
buffer (PBS, TWEEN (registered trademark) 20 (0.05%
v/v)) so as to give a total amount of about 25 μl.

Preparation of sample

Serum was used in this study. One hundred
microliters of each serum dilution was prepared by
diluting the serum at 1:100 to 1:1000 with a reaction

buffer (PBS (pH 7.4 ± 0.1), Tween (registered trademark) 20 (0.05% v/v), and bovine serum albumin (BSA) 10 mg/ml).

Assay of anti-peptide antibody

A biotinylated goat anti-human IgG (γ-chain specific) diluted with the reaction buffer was used as a secondary antibody. Streptavidin (1 mg/ml) labeled with PE (phycoerythrin) (SRPE) was diluted to 20 μl (1/50 dilution) with the above-mentioned reaction buffer and used as a fluorescent dye-labeled streptavidin.

One hundred microliters of the washing buffer was put in each well of a filter plate and then removed by aspiration. This washing step was carried out twice. Then, a 96-well filter plate containing 25 μl of the above-mentioned bead mixture in each well was washed with the washing buffer twice. After the washing, 100 μl of the sample was added to all wells. The filter plate was covered and shaken by a plate shaker (300 rpm) in the dark for 2 hours at room temperature and aspirated. Then, 100 μl of the washing buffer was put in each well and removed by aspiration. This washing step was carried out three times.

Then, 100 μl of the biotinylated secondary antibody, i.e., biotinylated goat anti-human IgG (γ-chain specific), was added to each well and the filter

plate was covered and shaken by a plate shaker (300 rpm) in the dark for 1 hour at room temperature. After aspiration, 100 μl of the washing buffer was put in each well and removed by aspiration. This washing step was carried out three times.

Subsequently, 100 μl of SRPE was added to each well and the filter plate was covered and shaken by a plate shaker (300 rpm) in the dark for 30 minutes at room temperature. After aspiration, 100 μl of the washing buffer was put in each well and removed by aspiration. This washing step was carried out three times. Thereafter, 100 μl of the washing buffer was added to each well and shaken by a plate shaker (300 rpm) for 2 to 3 minutes, and then 50 μl of the sample thus obtained was assayed by a fluorescence flowmetric system. The results are shown in Figs. 31 and 32 and Table 5.

Table 5

No	Peptide name	Alias	Region	Sequence	SEQ ID NO	HCV patients (n = 10)		Healthy donors (n = 10)	
						Positive		Positive	
						n	%	n	%
4	1bA24-717	E2-717	E2	EYVLLLFLL	8	6	60	2	20
14	1bA24-1716	NS4B-1716	NS4B	PYIEQGMQL	16	6	60	1	10
18	1bA24-885	NS2-885	NS2	IFTITKILL	20	9	90	2	20
39	1bA24-711	E2-711	E2	SFAIKWEYVL	38	6	60	2	20

Example 5: Treatment of HCV infection using peptide

vaccine derived from hepatitis C virus

An anti-virus effect of a vaccine comprising a

peptide derived from hepatitis C virus of the present

invention was examined in HCV-infected HLA-A2$^+$ or

HLA-A24$^+$ subjects. All the subjects were infected with

HCV-1b and showed no response to a treatment with

interferon and ribavirin. Peptides derived from

hepatitis C virus, C-35, NS5A-2132, E2-488, E1-213 and

NS3-1081 were synthesized, purified and stored as

lyophilized powder under GMP compliance. The C-35,

NS5A-2132, E2-488, and NS3-1081 peptides were dissolved

in a small amount of DMSO (1 mg/10 to 25 µl) and E1-213

was dissolved in 7% sodium hydrogen carbonate solution

for injection (Meylon) (1 mg/15 µl). Each solution was

diluted with physiological saline for injection (1 to

2 mg/ml) and sterilized by filtration through 0.22 µm

filter. Each of the resulting solutions was mixed with

an equal amount of an adjuvant (Montanide ISA-51), to

prepare emulsified injection solutions. C-35 injection

solution was administered to 3 subjects of HLA-A2$^+$ and

NS5A-2132 injection solution was administered to 5

subjects of HLA-A24$^+$. The peptide was administered

every two weeks by injecting the emulsion containing 0.3

mg of the peptide per dose into the lateral region. The

level of HCV RNA and the values of GOT, GPT, γ-GTP and AFP were monitored continuously.

The results are shown in Fig. 33 and Fig. 34. As obvious from these drawings, the level of HCV RNA was notably decreased after the vaccine administration was initiated in the majority of the subjects assayed, demonstrating that the peptides of the present invention are effective as an anti-HCV vaccine.

Industrial Applicability

The peptide derived from hepatitis C virus according to the invention contains an HLA-binding motif in its sequence. it is recognized by an antibody reactive to hepatitis C virus, and also has a property of being recognized by an HLA-A2- or HLA-A24-restricted cytotoxic T cell.

Accordingly, the peptide derived from hepatitis C virus of the invention could be an effective vaccine for a disease attributable to HCV infection.